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Interactions Between the Flk-1 Receptor, Vascular Endothelial Growth Factor, and Cell Surface Proteoglycan Identified with a Soluble Receptor Reagent

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Fetal liver kinase-1 (Flk-1) is a transmembrane tyrosine kinase that was identified in endothelial cells and populations of cells enriched in hematopoietic progenitors. To characterize the interaction of Flk-1 with potential ligands the receptor extracellular domain was genetically fused to an alkaline phosphatase (AP) tag. A soluble ligand for Flk-1 was identified in the supernatants of numerous mesenchymal cell lines by co-immunoprecipitation with the Flk1-AP fusion protein. This polypeptide was shown by N-terminal sequencing to be vascular endothelial growth factor (VEGF). Receptor-AP fusion proteins can thus be used to identify soluble ligands as well as transmembrane ligands, and this approach is therefore likely to be widely applicable to many types of orphan receptor. The Flk1-AP soluble receptor was also found to bind to cell surfaces, showing two apparent classes of binding site with different affinities. This interaction could be reconstructed by introducing a VEGF expression plasmid into cells. These results indicate that VEGF presented at the cell surface can bind to the Flk-1 receptor, and could mediate a direct cell-cell interaction. The Flk1-AP fusion protein was also found to bind heparin, implying that ligand binding by the Flk-1 receptor may involve a three way interaction between the Flk-1 receptor, VEGF, and heparin-like cell surface proteoglycans.

KEYWORDS: receptor tyrosine kinase, heparin, alkaline phosphatase

INTRODUCTION

Cell surface receptors with an intracellular tyrosine kinase domain have powerful effects on proliferation and other aspects of cell behavior. When activated by mutation they can act as potent oncogenes, and they have important roles in normal physiology and development (Schlessinger and Ullrich, 1992). In addition to the receptor tyrosine kinases with known ligands, many additional receptor-like tyrosine kinases without known ligands have been identified, mostly by approaches based on the sequence conservation of the enzymatic tyrosine kinase domain. More than twenty of these orphan receptors are currently without known ligands, and it is likely that the identification of those ligands will make an important contribution to our understanding of cell-cell signaling.

The Flk-1 receptor was identified by polymerase chain reaction of mRNA from populations of mouse fetal liver cells highly enriched for primitive hematopoietic progenitors (Matthews et al., 1991). The Flk-1 receptor, and an apparent human homolog, KDR, was also found to be expressed in vascular endothelial cells (Terman et al., 1991; Millauer et al., 1993; Quinn et al., 1993). Structurally, the Flk-1 receptor has an extracellular region containing seven immunoglobulin-like domains, placing it in a subfamily with two other receptors that show close sequence homology. Those receptors are flt, which was shown to be a receptor for vascular endothelial growth factor (de Vries et al., 1992) and flt-4 (Aprelikova et al., 1992; Galland et al., 1992), a receptor still without a known ligand.

One approach to identifying the ligands of receptor tyrosine kinases is to use the receptor

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extracellular domain as a soluble affinity reagent. We have previously described the use of a soluble form of the c-kit receptor for that purpose (Flanagan and Leder, 1990). The receptor extracellular domain was fused to placental alkaline phosphatase, providing the molecule with a tag that binds to available antibodies and also has an intrinsic enzyme activity that can be easily and sensitively traced. This reagent was used to detect the kit ligand as a transmembrane molecule expressed at the cell surface (Flanagan and Leder, 1990). Similar approaches based on soluble receptor fusion proteins have now also been used to identify other transmembrane ligands at the cell surface (for example, Armitage et al., 1992; Lyman et al., 1993).

Here we have applied the soluble receptor approach to the Flk-1 receptor and shown that this approach can be used to identify not only transmembrane ligands, but also ligands that are matrix-associated or soluble. The Flk-1 receptor was found by this approach to bind with high affinity to VEGF, an interaction also described by others (Millauer et al., 1993; Quinn et al., 1993). The interaction was detectable in solution and also when VEGF was present at cell surfaces, where it is bound via cell surface proteoglycan. In addition, the soluble Flk-1 receptor itself was found to bind to heparin-agarose. These results suggest that a three-way functional complex may be formed between the Flk-1 receptor, VEGF, and specific heparin-like molecules at the surface of the ligand-presenting cell.

METHODS

Production of Flk1-AP Fusion Protein

The Flk-1 extracellular region cDNA was amplified by polymerase chain reaction to create a HindIII-BamHI fragment, which was inserted into HindIII-BglII cut APtag-1 vector (Flanagan and Leder, 1990). The resulting Flk1-AP fusion plasmid encodes the entire extracellular domain of Flk-1 joined at Glu-762, via a four amino acid linker (Gly-Ser-Ser-Gly), to the distinctively heat-stable secreted human placental alkaline phosphatase. A plasmid with the same vector sequences encoding unfused secreted alkaline phosphatase (SEAP) (Berger et al., 1988) was also produced for use as a control. Plasmids were linearized with ClaI and transfected with the marker plasmid pSV7neo into NIH-3T3 cells by calcium phosphate precipitation. One day after transfection, cells were distributed into 96-well plates and selected with 400 μg/ml G418. After 2 weeks, approximately 100 clones were screened for secretion of alkaline phosphatase activity by a colorimetric assay as described (Flanagan and Leder, 1990), except that L-homoarginine was omitted from all alkaline phosphatase assays here. Alkaline phosphatase activities are expressed here as OD units per hour (OD/hr), indicating the rate of hydrolysis of the chromogenic substrate p-nitrophenyl phosphate under the conditions used. One picomole of alkaline phosphatase protein corresponded to an activity of approximately 30 OD/hr.

Co-immunoprecipitation with the Flk1-AP Fusion Protein

For analysis of ^{35}S-labeled proteins in supernatants, cells in 10 cm plates were rinsed twice in methionine-free DMEM, then incubated with 200 μCi/ml ^{35}S protein labeling mix (New England Nuclear, NEG-072) in 4 ml of methionine-free DMEM containing 10% dialyzed calf serum. After 8 hr, supernatants were taken and concentrated 10 fold by ultrafiltration. 200 μl of concentrated supernatant was incubated for 90 min at room temperature with an equal volume of conditioned medium containing approximately 5 μg/ml Flk1-AP fusion protein. The Flk1-AP fusion protein was then immunoprecipitated by incubating on a rotator for 60 min with CNBr-Sepharose beads coupled to excess monoclonal antibody against human placental alkaline phosphatase (Cat. no. MIA 1801, Medix Biotech Inc., Foster City, CA) and washing the beads six times in modified RIPA buffer (0.5% NP40, 0.5% NaDOC, 0.025% SDS, 144 mM NaCl, 20 mM Tris-HCl pH 8.0). ^{35}S-labeled proteins were separated on 15% SDS-polyacrylamide gels, which were then fixed, treated with Enlightening (New England Nuclear), dried, and exposed to X-ray film.

To prepare proteins for microsequencing, 200 μl of CNBr-Sepharose was conjugated with 400 μg anti-AP antibody and was then incubated with a saturating amount of Flk1-AP conditioned medium for 1 hr at room temperature. The beads were then washed and treated with the crosslinking agent dimethylpimelimidate (Harlow and Lane, 1988). BMS-12 cells were grown in DMEM containing 1% bovine calf serum for 3 days. One liter of conditioned medium was collected and concentrated to

30 ml by ultrafiltration and was incubated with the 200 μl of Flk1-AP conjugated beads. After 1 hr at room temperature the beads were packed into a column, washed with 4 ml of modified RIPA buffer, 1 ml of 10 mM sodium phosphate pH 6.8, and proteins were then eluted with 1 ml of 100 mM glycine, pH 2.5. The eluted sample was concentrated by TCA precipitation, run on a 15% SDS-polyacrylamide gel, and transferred to a PVDF membrane (Matsudaira, 1987). The band of interest was cut out and gas-phase protein sequencing was performed as described (Tempst and Riviere, 1989).

Quantitative Assays of Flk1-AP Binding to Cell Surfaces or to Heparin Sepharose

Cell surface binding of Flk1-AP was measured essentially as described previously for Kit-AP (Flanagan and Leder, 1990). Plates of cells were washed with HBHA buffer (Hank's balanced salt solution with 1 mg/ml bovine serum albumin, 20 mM HEPES pH 7.0, 0.1% NaN_3) and then incubated for 90 min at room temperature with conditioned medium containing Flk1-AP fusion protein or SEAP protein as a control. For some experiments the conditioned medium was diluted with HBHA buffer. The cells were then rinsed six times with HBHA buffer, lysed, and assayed for alkaline phosphatase activity colorimetrically as described (Flanagan and Leder, 1990). Scatchard analyses of cell surface binding data were performed with the LIGAND program (Munson and Rodbard, 1980).

To test the effect of heparinase on binding of Flk1-AP to the cell surface, cells were incubated in DMEM without serum for 1 hr at 37°C with 0.25 units/ml of heparinase (a gift from Ibex Technologies, Montreal, Canada). To test binding of Flk1-AP to heparin-Sepharose, conditioned medium containing Flk1-AP or SEAP was diluted into 10 ml of 20 mM HEPES pH 7.0, 150 mM NaCl and was loaded onto a 1 ml heparin Sepharose FPLC column (HiTrap column, Pharmacia) by recirculating overnight. Salt gradient elution was performed at a flow rate of 0.5 ml/min with collection of 1 ml fractions.

RESULTS

The Flk1-AP soluble receptor fusion protein used in these studies is illustrated in Fig. 1A. When immunoprecipitated from the supernatant of transfected

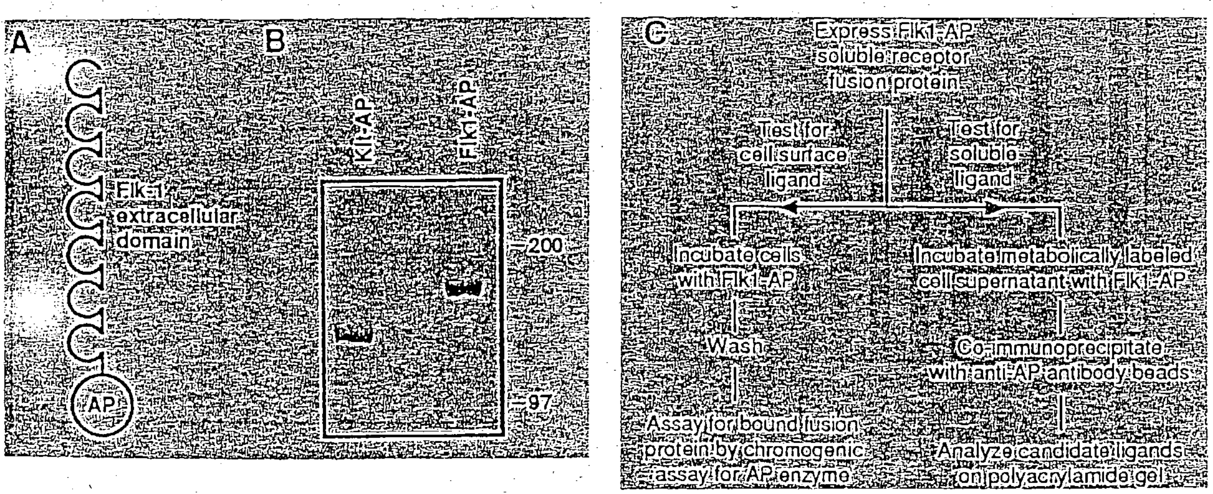

FIGURE 1. A soluble receptor affinity reagent used to screen for cell surface or soluble ligands. (A) The structure of the Flk1-AP soluble receptor fusion protein, consisting of the seven immunoglobulin-like domains of the Flk-1 extracellular region fused to a human placental alkaline phosphatase tag. (B) The Flk1-AP fusion reagent was produced by transfected NIH-3T3 cells as a single major polypeptide of the expected molecular weight. The supernatant of a transfected clone expressing high levels of alkaline phosphatase activity was immunoprecipitated with anti-AP beads and analyzed by electrophoresis on a 6% polyacrylamide gel followed by Coomassie blue staining. A Kit-AP fusion protein is shown for comparison (Flanagan and Leder, 1990). (C) Illustrates the strategy used to perform an initial screen of cell lines for either a cell surface ligand or a soluble ligand. To screen for a cell surface ligand (left side) the cells were treated with Flk1-AP fusion protein, washed and then tested for binding of the soluble receptor by a simple colorimetric assay for bound AP activity. To test for soluble ligand (right side) metabolically labeled supernatants from candidate cell lines were mixed with supernatant containing Flk1-AP fusion protein. The fusion protein, with any bound ligand, was then immunoprecipitated with anti-AP beads, and radiolabeled proteins were analyzed by gel electrophoresis.

cells, the fusion protein appears as a single prominent band with the expected apparent molecular weight of approximately 170 kDa (Fig. 1B). This fusion protein retained alkaline phosphatase enzyme activity, with a specific activity similar to that reported previously (Flanagan and Leder, 1990). Individual clones of transfected cells selected for secretion of high alkaline phosphatase activity produced approximately 5 $\mu g/ml$ of fusion protein in the supernatant.

The strategy used to screen cell lines for production of a Flk-1 ligand is summarized in Fig. 1C. As Flk-1 was implicated as a receptor that might function in hematopoietic progenitors and endothelial cells, we focused our initial ligand search on mesenchymal cell lines such as bone marrow stromal cells and embryonic fibroblasts, on the basis that such cells might be expected to support the growth of the receptor-bearing cell types. Figure 2 shows the result of a screen for cell surface binding to thirteen cell lines, including ten mouse bone marrow stromal lines (BMS and BMSC lines), one mouse embryonic fibroblast line (STO), one rat liver cell line (BRL 3A) and one mouse macrophage line (P388D1). The cells were treated with Flk1-AP to test for the presence of a candidate ligand and also with unfused SEAP as a control for background binding. In repeated experiments, each of the cell lines tested except P388D1 and BRL 3A showed cell surface binding of Flk1-AP that was several times higher than the SEAP control. Representative results are shown in Fig. 2.

As a biologically significant receptor-ligand interaction is expected to have a reasonably high affinity, a Scatchard analysis of the cell surface binding was performed by carrying out the binding assay with varying amounts of Flk1-AP. The binding data give a nonlinear Scatchard plot consistent with binding to two sites of different affinities on the cell surface (Fig. 3). This is in contrast to other AP tagged soluble receptors, such as the c-kit receptor, which give linear Scatchard plots when tested for binding to their cell surface ligands (Flanagan and Leder, 1990; and unpublished data). The dissociation constants for Flk1-AP binding to the cell surface calculated from repeated experiments were approximately 10^{-10} M for the higher affinity site and

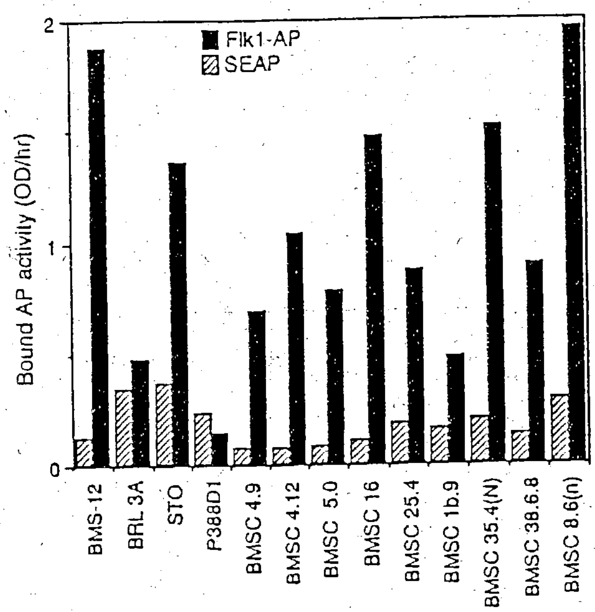

FIGURE 2. Binding of Flk1-AP soluble affinity reagent to the surfaces of cell lines. Cell lines in 10 cm dishes were incubated with 4 ml of conditioned medium containing Flk1-AP or SEAP as a control, each at 600 OD/hr per ml, for 1 hr at room temperature, then the cells were washed and analyzed for bound alkaline phosphatase activity. Cell lines denoted BMS or BMSC are mouse bone marrow stromal lines (M.-K.C., J.G.F., and N. Weich and W. Benjamin, Hoffman LaRoche Inc.), BRL 3A is a rat liver line, STO is a mouse embryo fibroblast line, and P388D1 is a mouse macrophage line.

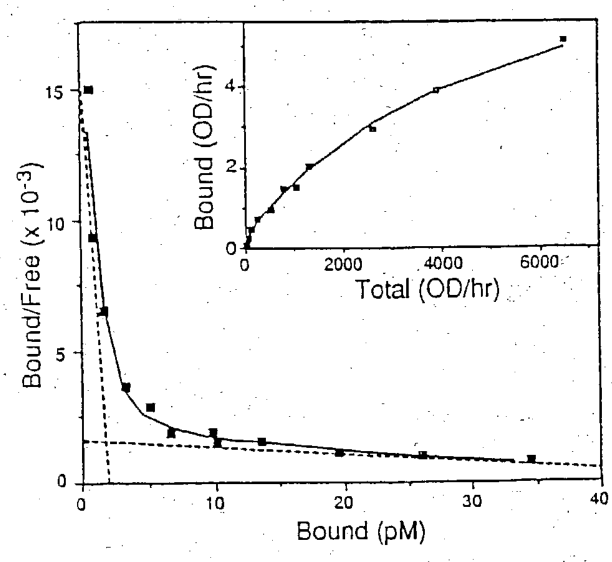

FIGURE 3. Scatchard analysis of Flk1-AP binding to the surface of BMS-12 cells. BMS-12 hematopoietic stromal cells in 10 cm plates were incubated with varying concentrations of Flk1-AP fusion protein in 4 ml of medium, and were then washed and assayed for bound alkaline phosphatase activity. The inset graph shows the measured alkaline phosphatase activities. The same data are shown as a Scatchard plot with a curve calculated for two cell surface binding sites of different affinities. The results of this experiment indicate approximately 170,000 sites per cell with a dissociation constant of 3.5×10^{-8} M and 6000 sites per cell with a dissociation constant of 1.3×10^{-10} M.

approximately 10^{-8} M for the lower affinity site, and the numbers of sites per cell were approximately 5000 and 100,000 respectively (Fig. 3). The dissociation constant of the higher affinity site is similar to recent estimates of the affinity of ^{125}I-labeled VEGF binding to Flk-1 receptor expressed on cell surfaces (Millauer et al., 1993; Quinn et al., 1993).

In parallel with the screen for cell surface ligands, we also screened cell lines for secretion of soluble Flk-1 ligands by a co-immunoprecipitation procedure (Fig. 1C). Two polypeptides with apparent molecular weights of approximately 19 and 23 kDa were detected prominently in supernatants of all the cell lines tested, including those shown in Fig. 4 as well as the 9 additional hematopoietic stromal cell lines described in Fig. 2. These bands were absent from controls where unfused SEAP was substituted for the Flk1-AP fusion protein (Fig. 4A). Experiments where the amount of Flk1-AP fusion protein in the co-immunoprecipitation reaction was varied indicated that the affinity of the soluble receptor for these polypeptides was approximately in the nanomolar range, consistent with a biologically significant receptor-ligand interaction and with the results obtained from the cell surface binding experiments.

The yield of the candidate Flk-1 ligand polypeptides in co-immunoprecipitation experiments was several nanograms per ml of supernatant, as judged by silver staining of gels (data not shown). This implied that amino acid sequence information could be obtained from a moderate amount of conditioned

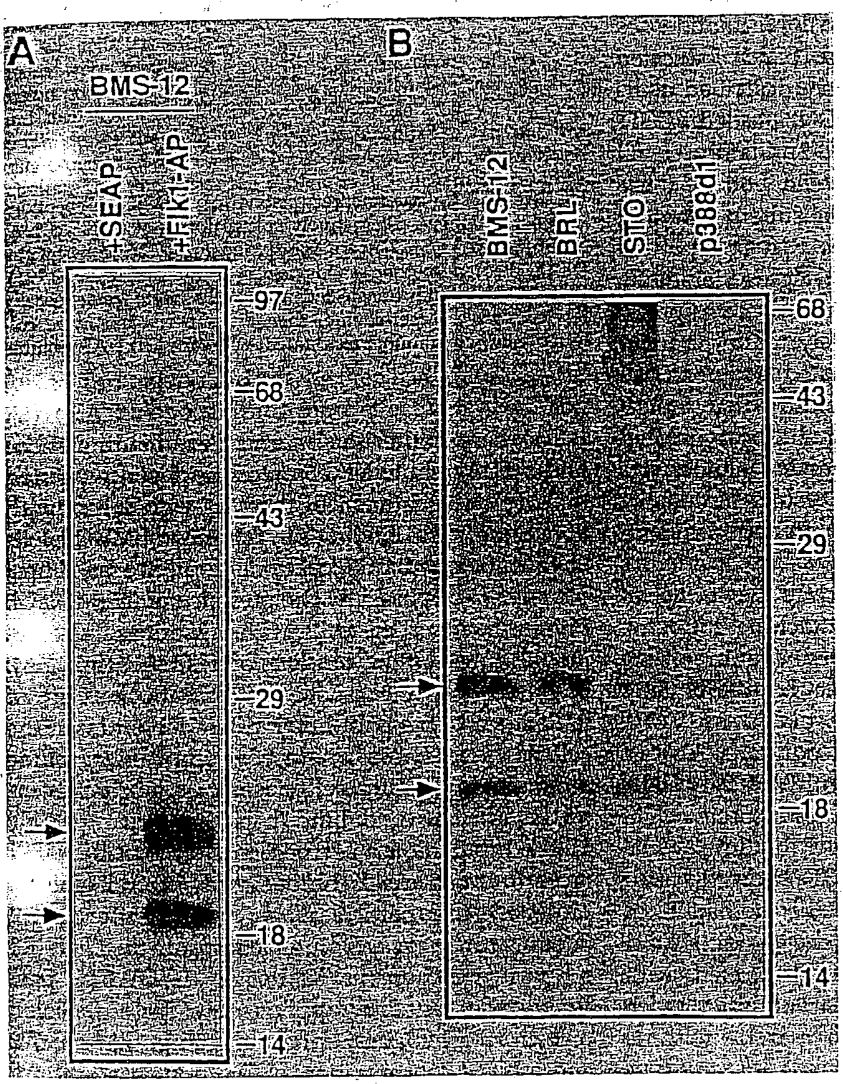

FIGURE 4. Co-immunoprecipitation of candidate ligand polypeptides with Flk1-AP. (A) BMS-12 bone marrow stromal cells were labeled with ^{35}S-methionine, then the supernatant was collected and treated with Flk1-AP fusion protein or SEAP as a control, followed by immunoprecipitation with anti-AP beads. Labeled proteins were then analyzed on a 15% polyacrylamide gel. (B) Supernatants from a variety of cell lines were co-immunoprecipitated with Flk1-AP, including a bone marrow stromal line (BMS-12), a liver cell line (BRL 3A), an embryonic fibroblast (STO), and a macrophage line (P388D1).

medium. One liter of supernatant from the BMS-12 cell line was therefore concentrated and co-immunoprecipitated, yielding approximately 2 μg of each of the candidate ligand polypeptides. After blotting onto a PVDF membrane, the 23 kDa band was subjected to N-terminal peptide microsequencing. The first nine amino acids of the resulting sequence were found to match the N-terminal sequence (APTTEGEQK) predicted from the cDNA of murine VEGF (Breier et al., 1992; Claffey et al., 1992).

To confirm binding of VEGF to the Flk-1 receptor, murine VEGF cDNAs were isolated by polymerase chain reaction (PCR). BMS-12 and two other lines (STO and P388D1) that express the 19 and 23 kDa co-immunoprecipitated polypeptides (Fig. 4) were tested by PCR and were each found to yield two prominent amplified bands visible by agarose gel electrophoresis (data not shown). Nucleotide sequencing of the cDNAs in these bands indicated that they correspond to previously described alternatively spliced forms of VEGF cDNA, called VEGF-1 and VEGF-2 (Breier et al., 1992; Claffey et al., 1992). When expressed in COS cells, both of these cDNAs yielded polypeptides in the supernatant that co-immunoprecipitate with Flk1-AP fusion protein (Fig. 5). The apparent molecular weights yielded by the VEGF-1 and VEGF-2 constructs were approximately 23 and 19 kDa respectively, corresponding in size to the two bands precipitated from BMS-12 (Fig. 5). These results further confirm that the 19 and 23 kDa polypeptides co-immunoprecipitated from BMS-12 and other cell lines are almost certainly VEGF polypeptides. The results also show that the VEGF-1 and VEGF-2 polypeptides both bind to the Flk-1 receptor.

It is known that VEGF can attach to the surface of expressing cells, and that this attachment can be eliminated by herparinase treatment (Ferrara et al., 1992). It therefore seemed possible that the binding of Flk-1AP to cell surfaces is mediated by VEGF associated with cell surface proteoglycans. To test this, COS cells were transfected with VEGF-1 cDNA and tested for Flk1-AP binding. Untransfected COS cells showed low levels of Flk1-AP binding, while cells expressing transfected VEGF showed much higher levels of binding (Fig. 6). Like the data from the BMS-12 stromal cell line, the binding data from the transfected COS cells are consistent with binding to two sites of different affinities, with apparent dissociation constants comparable to those measured for BMS-12 (Fig. 6).

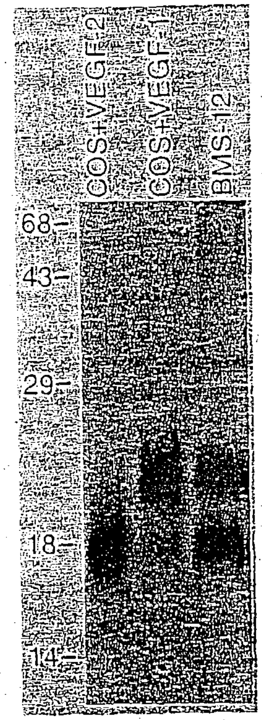

FIGURE 5. Co-immunoprecipitation of VEGF polypeptides with Flk1-AP. Two alternatively spliced forms of VEGF cDNA, VEGF-1 and VEGF-2, were transfected into COS cells by the DEAE dextran method as described (Flanagan et al., 1991). BMS-12 cells and transfected COS cells were metabolically labeled with ^{35}S-methionine, then the supernatants were collected and co-immunoprecipitated with the Flk1-AP soluble receptor fusion protein.

To assess further the nature of the binding of the Flk1-AP protein to VEGF on cell surfaces, we tested the effect of treatment of the cells with salt or with heparinase. The binding of Flk1-AP to BMS-12 cells or to transfected COS cells was found to be almost completely inhibited by the presence of 0.6 M NaCl. Pretreatment of the cells with heparinase also removed most of the binding of Flk1-AP (Fig. 7A). These results are consistent with an involvement of ionic interactions with heparin-like molecules at the cell surface. These interactions probably include binding of VEGF to cell surface proteoglycans, as VEGF is known to bind heparin (Ferrara et al., 1992). In addition it seemed possible that the Flk-1 receptor might itself bind directly to cell surface proteoglycans, particularly in view of the recent demonstration of an interaction of the FGF-R1 receptor with heparin (Kan et al., 1993). This possibility was tested by applying the Flk1-AP fusion protein to a heparin-Sepharose column. At pH 7.0 and a NaCl concentration of 150 mM, the Flk1-AP

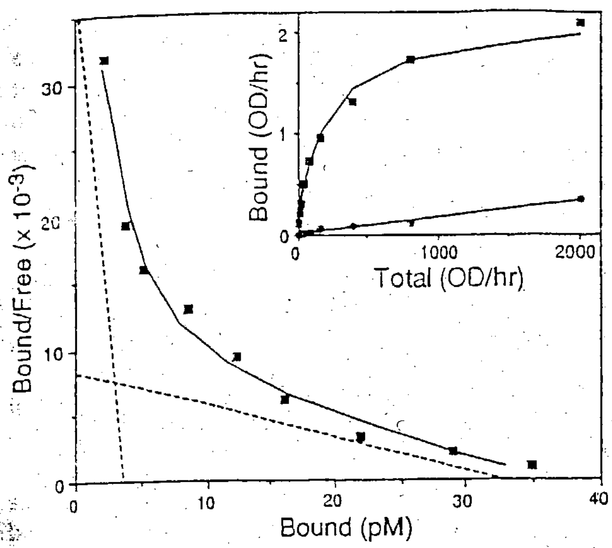

FIGURE 6. Scatchard analysis of Flk1-AP binding to the surface of COS cells transfected with VEGF cDNA. COS cells in 6 cm plates were treated with varying concentrations of Flk1-AP fusion protein in 2 ml of medium. The cells were then washed and assayed for bound alkaline phosphatase activity. The inset graph shows the measured alkaline phosphatase activities for cells transfected with VEGF-1 cDNA (squares) or vector alone (circles). The same data are shown as a Scatchard plot with a curve calculated for two cell surface binding sites of different affinities. The results of this experiment indicate approximately 135,000 sites per cell with a dissociation constant of 1.1×10^{-8} M and 29,000 sites per cell with a dissociation constant of 3.1×10^{-10} M.

fusion protein bound to the heparin column (Fig. 7B). SEAP alone did not bind effectively (Fig. 7B), consistent with the low isoelectric point of placental alkaline phosphatase which would give it a net negative charge at pH 7.0. The Flk1-AP fusion protein was also found not to bind to unconjugated Sepharose (data not shown) indicating that it binds to the heparin moiety of the heparin-Sepharose matrix. Bound Flk1-AP fusion protein was eluted from the heparin-Sepharose column by NaCl concentrations of approximately 0.3 M (Fig. 7B). The interaction of Flk1-AP with the heparin column may be direct or might be mediated by other molecules present in the conditioned medium. However, it is unlikely that the Flk1-AP binding is mediated by VEGF because the Flk1-AP concentration in the conditioned medium is much higher than the expected concentration of VEGF. The results therefore suggest that binding of the Flk-1 receptor extracellular domain to heparin-like molecules may be involved in the formation of a three-way functional complex between the Flk-1 receptor, heparin-like components and VEGF.

DISCUSSION

Flk-1 is one of a large number of receptor tyrosine kinases that were identified by the nucleotide sequence conservation of the kinase domain, but initially had no known ligands. We and others have previously used soluble versions of cell surface receptors to identify ligands that are transmembrane molecules (for example, Flanagan and Leder, 1990; Lyman et al., 1993). However, it has been less clear whether the soluble receptor approach could also be generally applied to ligands that are soluble. Here we have used a receptor-AP fusion protein to identify a soluble ligand for the Flk-1 receptor. The Flk1-AP reagent was used in a co-immunoprecipitation procedure to identify a candidate ligand in the supernatants of numerous mesenchymal cell lines. Co-immunoprecipitation from moderate amounts of supernatant allowed the isolation of a sufficient yield of the ligand for peptide microsequencing, showing that this ligand is VEGF. This growth factor was also shown by others to bind Flk-1 or its human homolog KDR, and was found to activate Flk-1 kinase activity (Terman et al., 1992; Millauer et al., 1993; Quinn et al., 1993). These results support the idea that the soluble receptor affinity approach is likely to be of general utility for the identification and characterization of a wide variety of different types of ligand, whether they are transmembrane, matrix-associated or soluble.

Placental alkaline phosphatase serves as a useful fusion protein tag. The availability of antibodies against placental alkaline phosphatase makes it straightforward to use procedures such as co-immunoprecipitation. Moreover the marker enzyme activity of the tag allows the fusion protein to be traced quantitatively by simple chromogenic assays without the necessity of purification, radioactive labeling, or the use of secondary reagents. We find that detection using the enzyme activity of AP fusion proteins is compatible with a variety of applications including quantitative receptor-ligand binding studies, *in situ* staining for ligands, and library screening. The sensitivity of detection can be at least comparable to other methods, such as the use of purified and ^{125}I labeled reagents (Flanagan and Leder, 1990; Flanagan et al., 1991; Ornitz et al., 1992, this paper, and unpublished data).

VEGF was originally discovered as a secreted polypeptide that affects endothelial cell growth and vascular permeability, and it has been implicated as an important factor in angiogenesis associated with

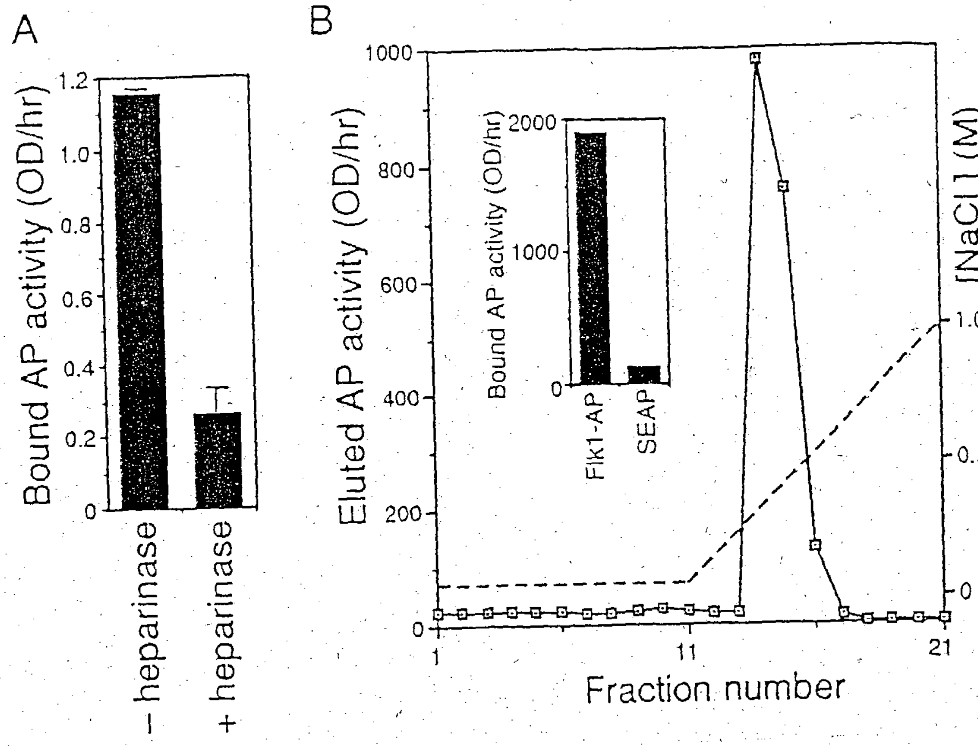

FIGURE 7. Role of heparin-like molecules in the interactions of Flk1-AP. (A) Effect of heparinase on the binding of Flk1-AP to VEGF on cell surfaces. COS cells transfected with VEGF-1 cDNA were incubated with or without heparinase, then were treated with Flk1-AP (500 OD/hr/ml), washed and assayed for bound AP activity. Each bar indicates the mean of two determinations, and the error bars indicate the difference between the two. (B) Binding of Flk1-AP to heparin-Sepharose. Approximately 2500 OD/hr (12.5 μg) of Flk1-AP fusion protein or the same activity of SEAP protein was loaded onto a 1 ml heparin-Sepharose FPLC column. The inset histogram shows total binding of Flk1-AP or SEAP. A salt gradient elution profile of Flk1-AP is also shown (solid line). After Flk1-AP binding the column was washed with 10 ml of buffer containing 50 mM NaCl, and bound proteins were then eluted with a gradient of NaCl (broken line).

both tumor formation and normal development (Folkman and Klagsbrun, 1987; Breier et al., 1992; Claffey et al., 1992; Ferrara et al., 1992; Plate et al., 1992; Shweiki et al., 1992; Kim et al., 1993; Millauer et al., 1993; Quinn et al., 1993; Millauer et al., 1994). In mice, two major alternatively spliced forms of VEGF mRNA and an additional minor form have been identified (Breier et al., 1992; Claffey et al., 1992). In this study we found that Flk-1 binds the protein products of both major alternative spliced forms of VEGF, VEGF-1 and VEGF-2. As Flk-1 is a receptor for VEGF and is expressed in endothelial cells or their progenitors from early stages of development, it is likely that the interaction of VEGF with the Flk-1 receptor has an important role in the control of angiogenesis (Millauer et al., 1993; Quinn et al., 1993; Millauer et al., 1994). The expression of Flk-1 in populations of cells highly enriched for primitive hematopoietic progenitors is also suggestive of possible roles for this receptor in hematopoiesis (Matthews et al., 1991), and could be consistent with the possible existence in the embryonic blood islands of a common progenitor for endothelial cells and hematopoietic stem cells (Risau, 1991).

Receptor-ligand interactions are typically studied at the surface of the receptor-bearing cell, although the interactions of receptors, growth factors and accessory molecules at the surface of the ligand-presenting cell may also be an important determinant of biological activity. The use of soluble receptor fusion proteins, as described here, allows a characterization of such interactions. We find that the soluble Flk-1 receptor binds VEGF-1 on the surfaces of expressing cells, without treatment to release the factor into solution. These results indicate that when VEGF is associated with proteoglycans or other molecules at the surface of the expressing cell, it is not sequestered from direct

binding to its receptor and is thus likely to be capable of mediating a direct cell-cell interaction. Interestingly, the binding of soluble Flk-1 to VEGF on the cell surface produces a non-linear Scatchard plot consistent with binding to two classes of site with different affinities. This could be due to an involvement of heparin-like molecules in the interaction. For example one model would be that the lower affinity site could represent a simple interaction between VEGF and Flk-1 only, while the higher affinity site could be produced by a further stabilization of this complex by heparin-like molecules interacting with both VEGF and Flk-1. Further support for this model comes from the finding that the soluble Flk-1 receptor can itself bind to heparin, suggesting the possibility of a direct interaction between the Flk-1 receptor and heparin-like molecules at the cell surface or in extracellular matrix. This model is also consistent with a recent report showing that the interaction of soluble VEGF and Flk-1 can be modulated by soluble heparin (Tessler et al., 1994).

Our results suggest that there may be some cell type specificity in the ability to present VEGF. For example, the P388D1 and BRL 3A cell lines were found to express VEGF polypeptides, but little or no Flk1-AP fusion protein bound to the surface of those cell lines. Specific cell surface proteoglycans may therefore be required to present VEGF on the cell surface. In this regard, it is of interest that the P388D1 line was recently shown to express a specific subset of cell surface proteoglycans (Yeaman and Rapraeger, 1993). Specific interactions with accessory proteoglycans might play an important role in modulating the activity of VEGF and could represent potential targets for therapeutic intervention.

In several respects VEGF is similar to basic fibroblast growth factor. They are both heparin binding growth factors and have angiogenic activity *in vitro* and *in vivo* (Folkman and Klagsbrun, 1987; Ferrara et al., 1992; Plate et al., 1992; Shweiki et al., 1992; Kim et al., 1993; Millauer et al., 1994). Heparin-like molecules are known to promote high affinity functional binding between both factors and their receptors (Klagsbrun and Baird, 1991; Rapraeger et al., 1991; Yayon et al., 1991; Bernfield et al., 1992; Gitay-Goren et al., 1992; Ornitz et al., 1992). A receptor-heparin interaction was observed here for Flk-1, and has also been reported for the fibroblast growth factor receptor FGF-R1 (Kan et al., 1993). The specific spatial localization of angiogenic factors on the cell surface or extracellular matrix and the effect of such interactions on the ability of their receptors to recognize these factors may play important roles in the control of angiogenesis.

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AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No. 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-7

referred to in the Statutory Declaration

of Peter Adrian Walton Rogers

made before me

DATED this 12th Day of November, 2001

(Signature of Witness)

Medical Practitioner